# THE ROLE OF PAR 1 (PROTEASE-ACTIVATED RECEPTOR 1) IN THE MALIGNANT AND PHYSIOLOGICAL INVASION PROCESSES

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Protease Activated Receptors (PARs) are G-protein - coupled membrane proteins consisting of four family members, all which are activated via proteolytic cleavage. PAR1, the first prototype of the family, plays a central role in breast carcinoma invasion and metastasis. The criteria of genes that are part of the invasive program were demonstrated also in a physiological invasion system of the placenta where PAR1 is exclusively expressed during the time limited invasion process and completely shuts - off thereafter. PAR1 induced invasion is mediated partly by increased adhesion to extracellular matrix components and accompanied by the cytoskeletal re-organization of F-actin. Activation of PAR1 increased the phosphorylation of focal adhesion kinase (FAK) as also paxillin and induced the formation of focal contact complexes. While the differential expression of PAR1 is observed in a direct correlation to the metastatic potential, activation of PAR1 is essential for avb5 recruitment and integrin activation (since a truncated version of PAR1 lacking the entire cytoplasmic region failed to co-precipitate avb5). PAR1 has been also assigned as a novel oncogene that promotes growth transformation in NIH 3T3 cells and loss of anchorage-dependent growth. We prepared now mice carrying an MMTV LTR – driven Par-1 transgenes specifically overexpressed in the mammary gland. Analysis of whole mount glands of virgin human Par1 (hPar1) +/- mice, showed enhanced complexity of alveolar side branching as compared with normal virgin glands. A striking ductal side branching, budding from preexisting ducts was observed in pregnant mice of hPar1 overexpressing glands. This phenotype is precociously reminiscent of the effect of several oncogenes in the mouse breast. In parallel, we show that PAR1 is capable of recruiting blood vessels in vivo as demonstrated in a Par1 “Tet-on” inducible system and Matrigel plug assay. Our approach involving the combined analyses of tissue specific PAR1 transgenes, biopsy specimens and established cell lines may help elucidate the molecular mechanism of PAR1 in tumor metastasis and angiogenesis.

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# EXPRESSION OF RANK AND RANKL IS ALTERED IN INVASIVE CARCINOMA AND BONE METASTASIS OF BREAST CANCER

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Bone is the most common site of metastases by human breast cancer. Most breast cancers form osteolytic metastases, in contrast to tumors such as prostate cancer that form osteosclerotic metastases. Although some evidence suggests that formation of bone metastases by breast cancer cells is mediated by the increased osteoclastogenesis at the target site, a clear controversy exists whether formation of bone metastases is mediated by breast cancer cells directly or by stimulated osteoclasts. To test this we examined the expression of RANK and RANKL, two proteins critical to the bone remodeling signaling pathway, in invasive carcinoma of breast and bone metastases of breast. We observed increased expression of RANK and RANKL in the tumor cells from both the primary and metastatic lesions. Further, in the osteolytic metastases, breast tumor cells were seen directly in contact with the bone in the regions of osteolysis, without any osteoclasts in the vicinity. We suggest that overexpression of RANK and RANKL in breast cancer cells provides a growth advantage to the breast tumor cells, and the tumor cells appear to be directly responsible for the degradation of bone.

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# BIOCHEMICAL AND BIOPHYSICAL ANALYSIS OF THE MTS1 METASTASIS FACTOR

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Mts1, a member of the S100 family of calcium-binding proteins, has a strong causal link with breast cancer metastasis. Mts1 protein levels are higher in malignant human breast tumors than in benign tumors and correlate strongly with poor patient survival. Furthermore, mts1 expression correlates with increased cellular motility, suggesting that mts1 may promote the metastatic process by enhancing the invasive properties of tumor cells. To investigate the functional and cellular activities of mts1, we determined the structure of the calcium-free mts1 using multidimensional heteronuclear NMR spectroscopy. The apo-mts1 contains four alpha-helices, five loops and two short beta-strands. Similar to other S100 proteins, mts1 has two globular domains each consisting of a helix-loop-helix calcium binding motif. Biochemical analyses demonstrate that mts1 has a 9-fold higher affinity for myosin-IIA filaments than for myosin-IIB filaments and also shows a weak calcium-dependent interaction with actin. In addition, at a 1:1 stoichiometry mts1 inhibits the assembly of myosin-IIA filaments and promotes the disassembly of preformed myosin-IIA filaments, but has no effect on myosin-IIB filaments. These findings demonstrate that mts1 modulates the monomer-polymer equilibrium of myosin-II, but also indicate that mts1 may specifically and preferentially regulate myosin-IIA activity in vivo. These in vitro data demonstrate a direct biochemical linkage between mts1 and the actomyosin cytoskeleton, and support the hypothesis that mts1 exerts its effect on metastasis through modulation of cellular motility.

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# EVIDENCE THAT THE ESTROUS CYCLE CAN INFLUENCE ORGAN-SPECIFIC METASTASIS

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The idea that timing of surgery for breast cancer during a pre-menopausal woman's menstrual cycle can impact on survival has been a controversial one. Some clinical studies have shown improved survival when surgery was performed during the luteal phase of the cycle, while other studies failed to find this association. We previously showed that gene expression patterns varied in breast tumors resected in different menstrual phases, and it also is known that normal tissues respond to cyclic hormones. Tumor cells shed at surgery at different menstrual phases thus might vary in their ability to form metastases, depending on hormone-responsive variations in host tissues and/or tumor cells. Our goal is to develop animal models in which to test this idea.

To mimic the shedding of tumor cells during surgery, we injected cells into mice intravenously (tail vein) during either proestrus or metestrus (determined by vaginal cytology). While human and murine reproductive cycles differ in many respects, mouse proestrus corresponds to the follicular phase and metestrus to the luteal phase. Initially, as a control, we used a non-breast cancer cell line, murine B16F10 melanoma cells. Surprisingly, we found unexpected differences in the organ specificity of metastases from these cells. While there was no difference in metastatic burden in the lung, 32% of mice injected in metestrus (6/19) had prominent ovarian metastases while mice injected in proestrus (0/17) had none ($p = 0.036$). The presence of ovarian or other extrapulmonary metastases did not correlate with the metastatic burden in the lungs in either group, suggesting that the extrapulmonary metastases were not the result of secondary metastasis.

These novel finding suggest that a fluctuating hormonal milieu may differentially affect interactions of circulating tumor cells and secondary tissues in the establishment of metastases. While further work is necessary to characterize this phenomenon, these preliminary results provide an intriguing suggestion that the hormonal status at the time of entry of cancer cells into the blood stream can determine whether metastases form in specific organs. It is important that this phenomenon be studied further, to clarify the potential of relatively simple manipulations (scheduling surgery at defined menstrual phases, or hormone treatment at the time of surgery) to significantly improve the probability of survival for pre-menopausal women with breast (and perhaps other) cancers.

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# GAP JUNCTIONS AND BONE METASTASIS

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**Introduction** Gap junctions are membrane spanning channels that facilitate intercellular communication by allowing small signaling molecules to pass from cell to cell. Connexin (Cx) 43, a specific gap junction protein, suppresses the cancer phenotype of human mammary carcinoma cells and is correlated with breast cancer cell metastasis. Osteopontin (OPN) expression is related to cancer cell metastatic potential and we have previously reported an inverse relationship between Cx43 and OPN expression in bone cells. Additionally, Cx43 expression is undetectable in metastatic MDA-MB-435 breast cancer cells (435) whereas OPN and Cx32 expression is upregulated in 435 cells, relative to 435 cells expressing the metastasis suppressor gene BRMS-1 or non-metastatic breast epithelial cells Hs578Brt (Hs578). In this study we examined the hypothesis that restoring Cx43 expression in 435 cells would alter GJIC as well as Cx32, OPN and MMP-1 expression.

**Methods** 435 cells were transfected with a plasmid containing human Cx43 cDNA. Clones were screened by northern blot with a human Cx43 cDNA probe and high Cx43 mRNA profile clones were selected for further examination. 435, 435 cells transfected with Cx43 (435/Cx43$^+$), plasmid vector controls (435/pvcHY) and osteoblastic hFOB cells were examined for GJIC using a dual label dye transfer technique. Steady state levels of mRNAs for Cx43, OPN and Cx32 were quantified by real time RT-PCR. MMP-1 protein levels were quantified by ELISA.

**Results** Cx43 was detected in all 15 clones of 435/Cx43$^+$ while Cx43 was undetectable in all 12 clones of 435/pvcHY. Cx43 mRNA levels similar to 435/Cx43$^+$ were detected in hFOB cells and non-metastatic breast epithelial cells. OPN and Cx32 mRNA levels, and MMP-1 protein levels, were decreased in 435/Cx43$^+$ cells relative to 435 or pvcHY, but were still greater than in hFOB cells and Hs578. Homotypic (same cell type) GJIC between 435/Cx43$^+$ cells was increased 5 to 8.5 fold relative to GJIC between 435/pcvHY cells. Heterotypic (different cell type) GJIC between 435/Cx43$^+$ and hFOB cells increased 1.5 to 3 fold relative to that between 435/pvcHY and hFOB cells.

**Discussion** Restoring Cx43 expression in 435 cells decreased OPN and MMP-1 expression, both markers of highly invasive cancer cells, and Cx32 expression which, while expressed in metastatic 435 cells, we had previously shown is undetectable in metastasis suppressed 435/BRMS-1 cells and normal breast epithelial cells. Cx43 expression restored homotypic GJIC between 435 cells, a lack of which is associated with tumorigenic and metastatic potential, and increased heterotypic communication between breast cancer cells and osteoblastic cells. These results suggest an inverse relationship between Cx43 expression and metastatic potential of breast cancer cells. Furthermore, since breast cancer cells which do and do not express Cx43 appear to express different metastatic potentials and since heterotypic GJIC between these cells and osteoblastic cells differ, we suggest that an alteration in GJIC may be related to breast cancer cell metastasis to bone.

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# EFFICACY OF VITAMIN D COMPOUNDS TO MODULATE ER NEGATIVE BREAST CANCER GROWTH AND METASTASIS

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Metastatic spread of cancer continues to be the greatest barrier to cancer cure. In ER(+) breast cancer cells, the effects of 1,25(OH)2D3 are similar to those induced by anti-estrogens, where 1,25(OH)2D3 transcriptionally down-regulates ER expression. These finding have led to the hypothesis that in ER(+) breast cancer cells, the effects of 1,25(OH)2D3 may be related to disruption of estrogen mediated survival signals. If so, then sensitivity to 1,25(OH)2D3 mediated apoptosis could be reduced in estrogen independent breast cancer cells. We focused on the role of vitamin D3 compounds to activate the apoptotic pathway in ER(-) SUM-159PT cells and tumors and whether vitamin D3 compounds were capable of inhibiting the invasion of SUM-159PT cells. Our major findings are that 1,25(OH)2D3 induces apoptosis in the ER(-) negative SUM-159PT cells by disruption of mitochondrial function which is associated with bax translocation to mitochondria and cytochrome c release. Downstream events of the mitochondria triggered by 1,25(OH)2D3 included PARP (poly (ADP-ribose) polymerase) cleavage. Our in vivo data is the first to show apoptotic tumor regression of an ER independent breast cancer model system by the vitamin D3 analogue EB1089. When SUM-159PT tumor bearing nude mice are implanted with pellets designed to release 120pmoles EB1089 per day, a significant reduction in tumor volume is observed after 5 weeks of treatment with several mice showing complete tumor regression. The reduced growth of tumors from EB1089 treated mice is associated with characteristic apoptotic morphology, an increase in DNA fragmentation and a reduction in proliferation relative to control tumors. Our in vitro invasion assays demonstrate a dramatic reduction in invasive potential of SUM-159PT cells after exposure to 1,25(OH)2D3 and EB1089. These data demonstrate that vitamin D3 compounds may inhibit both growth of primary tumors and metastasis of breast cancer cells, and may in the future prove to be highly useful in the clinical setting, alone or in conjunction with other agents in the treatment of hormone-independent, metastatic breast cancer.

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# SERIAL ANALYSIS OF CIRCULATING TUMOR CELLS (CTCS) BY CK19 MRNA EXPRESSION AND SHED HER-2 EXTRACELLULAR DOMAIN (ECD) IN HER-2 POSITIVE METASTATIC BREAST CANCER PATIENTS TREATED WITH HERCEPTIN AND NAVELBINE (H&N)

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PURPOSE: To evaluate the feasibility of detecting CTCs by CK19 mRNA in patients with HER-2 positive metastatic breast cancer and correlate this with serum HER-2 and clinical behavior to H&N.

PATIENTS AND METHODS: Blood samples were collected from 55 women with HER-2 positive (IHC3+ and/or FISH+) metastatic breast cancer and 30 healthy female volunteers. Blood samples were collected at baseline, during the first cycle of H&N treatment at weeks 2, 3 and 5, and at each restaging. Peripheral blood mononuclear cells were isolated and enriched for epithelial cells by immunomagnetic selection (Dynabeads®). Plasma was used to measure shed HER-2 extracellular domain using commercially available kit (HER-2/neu Microtiter ELISA®; Oncogene Science). The presence of CK19 gene mRNA was determined by RT-PCR using the LCx probe system (Abbott diagnostics). Thirty healthy donor blood samples were assessed as controls.

RESULTS: CK19 mRNA was undetectable in 29/30 healthy controls. Of 23 patients with available samples for detection of epithelial cells, 9/23 (39%) were positive for CK19 expression at baseline. 7/9 responding patients became negative for CK19 after the first cycle. The remaining 2 patients who responded became negative after cycle 2. All primary progressors were negative at baseline; 1/3 became positive for CK19 at cycle 1, 2/3 remained negative. With subsequent cycles, all patients remained negative for CK19 although occasional low positive signals were seen. These samples are being assessed by quantitative RT-PCR for CK19. To assess possible reasons for lack of CK19 detection, in vitro studies were performed. No significant change in CK19 expression was observed in SKBR-3 cells treated with H&N for 24 and 48 hrs. These studies suggest that CK19 message is not downregulated by H&N. 48/55 patients had serial samples available for analysis of shed HER-2. 21/48 (44%) had elevated ECD using a cut-off value of 25 ng/ml. Comparison of serum HER-2 and CK19 from the same patient show consistent expression patterns of shed HER-2 and CK19.

CONCLUSIONS: Results of this study show that CK19 expressing CTCs are detectable by RT-PCR, and its expression correlates with circulating HER-2/ECD in patients treated with H&N. Detection of CK19 at progression is less reliable in this study, possibly because the threshold of detection of CTCs has not been reached. Additional experiments are underway to address this using a real-time quantitative CK19 assay as well as assays for additional markers including breast-specific markers.

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# EVALUATION OF HEPARANASE ACTIVITY IN BREAST CANCER

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Heparanase is a physiologically important enzyme that breaks down heparan sulfate polysaccharide found in mammalian extracellular matrix (ECM) and basement membrane (BM). The expression of heparanase activity by cancer cells has been linked to cancer invasiveness. The inhibition of heparanase activity has been indicated to reduce cancer cell invasion, cancer metastasis and the ability of cancer cells to stimulate the formation of new blood vessels to support tumor growth. We are exploring a research strategy combining new synthetic methodologies for the preparation of novel, inhibitors of breast tumor-associated heparanase with a strategy to screen for the inhibition of heparanase activity in breast cancer cells of varying metastatic ability.

To date, Hpa 1 mRNA and protein were expressed, as well as heparanase activity in a malignant breast cancer subline of MCF 10A cells. The mRNA expression was quantified by real-time RT-PCR. Proteins from concentrated media and lysate were collected and separated by molecular weight using a standardized Sepharose 4B column. Quantified Western analysis (ECF Western blot kit) was performed with primary antibody to hpa 1. Heparanase activity was measured by the degradation of 3H-HSPG. The positive control, neutrophil lysate, was also verified expressing hpa 1 mRNA, protein as well as heparanase activity. Fluorescein-labeled heparan sulfate was prepared and detection of its degradation by heparanase evaluated and compared to control, degradation with bacterial lyase. Novel saccharides have been prepared to identify structural features of these saccharides that afford substrate and inhibitor sequences against heparanase activity. The synthesis of these saccharides sequences employs a novel synthetic methodology and the evaluation of their ability to inhibit heparanase activity is in progress.

For cancer cells to spread, these cells must produce molecules that break down and degrade the barriers around the cell that would normally keep the cells from spreading. Heparanase is one of these important molecules. The findings in this study will provide a better understanding of heparanase expression at varied stages of breast cancer and will unveil recognition requirements for heparanase substrates and inhibitors.

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# LYMPHATIC CHANGES DURING EARLY TUMOR DEVELOPMENT

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Death from breast cancer is usually due to the spread of tumor cells from the primary site, where it can be effectively treated, to distant inaccessable or untreatable sites where metastases form. The movement of tumor cells to typical metaststic sites such as bone or brain must ultimately be via the blood circulation, but entry to the blood stream may occur indirectly via lymphatic channels draining the primary site or surrounding tissues. Spread via the lymphatic system is especially implicated in breast cancer, so in vivo models must be developed for studying the basic biology of the process and assessing therapies targeted to block it.

Intravital videomicroscopy (IVVM), based on established methods for studying hematogenous metastasis (Chambers et al, Adv. Cancer Res. 2000;79:91-121), was used to visualize subcutaneous lymphatic channels in mice. To develop this model, B16F1 murine melanoma cells, ($2\times10^5$) were injected intradermally to form midline tumors between the inguinal lymph nodes. Tissue on one side was used for IVVM and on the other for histology.

In untreated animals, afferent lymphatic channels were collapsed as seen by histology and were rarely evident by IVVM. Tumor formation, however, was accompanied by dilated afferent lymphatic vessels, seen by histology and IVVM. At high magnifications, IVVM clearly revealed endothelium and valves in dilated lymphatic vessels. Lymphatic capillaries originating in the region of the tumors had distinct valves at intervals (~500 μm) and were much wider (~ 40 μm) than blood capillaries. Lymphocytes were seen moving intermittently with fluid flow in these channels, advancing (~150 μm) during 3-5 s periods at 10-20 s intervals. Inflamed tissues immediately overlying the tumor contained closely spaced, highly anastomotic lymphatic channels with no valves. Many lymphocytes were seen within these vessels moving at a steady rate (~30 μm/s). Although lymphangiogenesis has been attributed to growth factors produced by tumor cells, sham injections (saline alone) also result in enlarged lymphatic channels. Thus, lymphatic changes accompanying tumor development may also be due to the physical effects of local damage or increased volume loading of the tissues.

These studies show that IVVM can serve as a powerful tool for quantifying the role of the lymphatic system in the spread of cancer.

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# MAMMARY GLAND STROMA CONTRIBUTES TO EPITHELIAL CELL NEOPLASIA

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Mammary gland development and function are driven by a complex network of signals between the stroma, the extracellular matrix and the epithelium, as well as hormones acting directly on these cells. The tissue organization field theory (TOFT) proposes that alterations of the reciprocal interactions between stroma and epithelium initiate the process of neoplastic transformation of epithelial cells. Our goal is to assess whether the primary target of the carcinogen N-nitroso-methylurea (NMU) is the epithelium, the stroma or both through a protocol of tissue recombination by transplanting mammary gland epithelial cells (MGEC) into mammary gland fat pads (MGFP) previously cleared of epithelium (CFP). Wistar-Furth rats are being used. The 4th and 5th MGFP were cleared at 21 days of age. One month later, these animals were divided into 4 groups:

| Group # | Stroma treatment | MGEC treatment | Pos. control | Neg. control |
|---|---|---|---|---|
| 1 | NMU (50mg/kg, i.p) | Vehicle | | |
| 2 | NMU | NMU | | |
| 3 | Vehicle (0.85% NaCl) | NM U | | |
| 4 | Vehicle | Vehicle | | |
| 5 | NMU | No | + | |
| 6 | Vehicle | No | | + |

One week later, vehicle-treated epithelial cells were transplanted into the CFP of Groups 1 and 4, and NMU-treated epithelial cells were transplanted into the CFP of Groups 2 and 3. Group 5 are intact virgin rats injected with NMU, and Group 6 are intact virgin rats injected with vehicle. Weekly palpations were done beginning 1 month after cell injection. Results: 87.5% of Group 1 and Group 2; 15.4% of Group 3; and 100% of Group 5 developed tumors. Groups 4 and 6 developed no palpable tumors. Whole mount preparations and histology confirmed the mammary tumor origin of the palpable lesion. Our results suggest that the stroma, rather than the epithelium, is the target of the carcinogen. This novel concept in carcinogenesis will provide clues to be applied to more rational study of breast cancer.

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# S-ADENOSYLMETHIONINE DECARBOXYLASE (SAMDC) OVEREXPRESSION REDUCES INVASIVENESS AND TUMORIGENICITY IN NUDE MICE OF MCF-7 BREAST CANCER CELLS

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Activation of the polyamine (PA) pathway may be involved in mammary carcinogenesis and breast cancer progression. Although considerable evidence supports an important role in breast cancer biology of increased activity of ornithine decarboxylase, the most proximal enzyme in the PA pathway catalyzing the formation of putrescine (Put), no information is available on the role of S-adenosylmethionine decarboxylase (SAMDC), the biosynthetic enzyme involved in the synthesis of the more distal PA, spermidine (Sd), and spermine (Sm). To address this issue, we generated SAMDC overexpressing MCF-7 breast cancer cells, whose PA profile is characterized by a selective accumulation of Sm associated with a profound suppression of Put and a moderate decrease in cellular Sd levels. SAMDC overexpression did not alter in a major way growth properties of MCF-7 cells in soft agar, either under basal conditions or in response to estrogens and antiestrogen administration. SAMDC-MCF-7 cells, on the other hand, exhibited a markedly reduced invasiveness in matrigel ($p=0.013$). Furthermore, they were less tumorigenic in nude mice. The odds for control clones to form tumors were 3.13 (C.1 1.2-8.2, $p = 0.0184$) higher than those for SAMDC clones. The odds ratios were identical in the absence and in the presence of estradiol. In addition, the growth rate of established tumors was slower for SAMDC than for control clones. Overall, no major differences were observed between control and SAMDC overexpressing cells with regard to activation of MAPK and STAT signaling in response to epidermal growth factor administration. Correlation of cellular PA profiles with biologic behavior indicates that the favorable phenotypic changes induced by SAMDC overexpression are primarily mediated by Put depletion. These findings provide important pre-clinical evidence for the design of optimal breast cancer treatment strategies targeting the PA pathway.

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# BONE-RESTRICTED SURVIVAL OF BREAST CANCER CELLS

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About 3/4 of recurrent breast and prostate cancers exhibit skeletal metastasis. While recurrent prostate cancers are typically (60-70%) restricted to skeletal sites, breast cancer recurrs as bone-limited disease in 20-30% of patients, the remainder manifest as extraskeletal as well as bone disease and a more fulminant clinical course. Competence of both prostate and breast cancers for culture or xenograft transplantation has been demonstrated most efficiently when whole tissue explants are used, with the growth of isolated cells rare for prostate cancers and low for breast cancers. Successful establishment of continuous breast cancer cell lines has been associated with high HER2/neu expression and high grade (or epithelial/mesenchymal metaplasia). We therefore suggest that current cell line models of breast and prostate cancer are representative of but a subset of such cancers, with bone-restricted cancers rare among available panels of cell lines.

This project is directed toward high efficiency propagation of cell culture-recalcitrant breast cancer cells from bone marrow biopsy, based upon a prediction that bone-restricted cancers may require initial culture and subsequent passage on normal bone stroma. Residual responsiveness to stromal cell products of existing cell lines may account for previous findings of bone cell responsiveness, and our initial studies have demonstrated that certain cell lines from cancers of breast (MB231 and MB435) and prostate (PC3 and LNCaP) are stimulated in colony forming efficiency (CFE) by coculture with normal bone-derived stroma. Other adenocarcinoma lines (MCF7, SKBr3, and DU145) are unaffected or inhibited. Evolving methods and apparatus for bone culture and for cancer-specific CFE determinations (i.e., cytokeratin antibody stained colonies) are facile and generally useful. The usefulness of the approach will be demonstrated for cocultured cancer/stroma cultures exposed to histone deacetylase inhibitors (HDI).

Initial mechanistic studies suggest that cells with a high sensitivity to cell death after HDI-treatment, whether plastic-cultured or bone stroma-cocultured, manifest moderate basal expression and refractoriness to HDI-induced increases in expression, of the cyclin/kinase modulators p21cip1 and/or p57kip2. These modulators of growth- and survival-regulatory cyclins have previously been shown to be regulated by extracellular matrix- liganded receptor/transducer complexes. Both basal and HDI-stimulated expression of these proteins are normalized (decreased and increased, respectively) by culture on bone stroma. Such measures provide insights into the molecular mechanism of support of breast cancer growth by stroma, as well as a rationale for selecting drugs that increase the relative response of cancer and normal cells to new therapeutic agents such as the HDI agents.

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# A NOVEL METHOD FOR STUDYING MAMMARY STROMAL EFFECTS ON HUMAN EPITHELIAL DIFFERENTIATION AND FUNCTION

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Stromal-epithelial interactions play key roles in mammary gland development. Unlike the mouse, investigation of the in vivo biology of human breast epithelium (huBrE) is limited to relatively few studies where normal huBrE cells have been transplanted into cleared fat pads of athymic nude mice (Sheffield, 1988), transplanted by injection (Dubois et al., 1987, Gusterson et al., 1984, Sheffield, 1988) and embedded in collagen gels transplanted to subcutaneous sites (Popnikolov et al., 1995, Yang et al., 1994).

We have modified the transplantation technique for huBrE in vivo by combining 6-8 human ductal organoids with 250,000 mouse or human mammary fibroblasts in a collagen gel, which is then grafted under the renal capsule of female nude mice. The graft is harvested one month after implantation and analyzed histologically.

The results appear greatly improved with more robust growth of the huBrE. Ducts produced by this method contain myoepithelial and luminal cells as shown immunohistochemically using the epithelial markers Keratin 8, Keratin 14, p63 and alpha-actin. Response to hormonal stimuli (estrogen and estrogen+progesterone) was also observed by an increase in branching morphogenesis and cellular proliferation and progesterone receptor (PR) induction. After hosts were made pregnant, grafts produced beta-casein and ducts underwent a morphological change comparable to the pregnant host mammary gland. We have utilized this model to assess the role of stromal estrogen receptor alpha (ER) by comparing proliferation response of huBrE grown in combination with wild type (WT) or ER knockout (KO) mammary fibroblasts and shown that stromal estrogen receptors are not required by human mammary epithelium to be estrogen responsive. We have also shown that mammary carcinoma-associated fibroblasts (Mg-CAF) cause abnormal development of huBrE. In MgCAF+huBrE recombinations, the hyperplastic ducts no longer exhibit lumen but instead grow as solid cords. We aim to use this finding to investigate the tumor inducing capability of a variety of mammary CAF.

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# VEGF PROMOTES THE GROWTH OF BREAST CANCER BRAIN METASTASES IN NUDE MICE

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Breast cancer is the second most common cause of brain metastases, after lung cancer. Relatively little is known about how the metastases form, and what phenotypes are characteristic of brain-metastasizing breast cancer cells. MDA-MB-231 human breast cancer cells were transfected with a gene encoding green fluorescent protein (MDA-231-GFP) and injected into the internal carotid artery of nude mice. The injection of 100,000 cells resulted in experimental brain metastases in 75% of mice, by 62 days. The expression of GFP was used as a marker to aid in the dissection of metastastic cells from the brain parenchyma, which were expanded in tissue culture, and then injected into the carotid artery. Three rounds of injection and recovery of tissue resulted in a variant termed 231-BR3. Mice injected with the 231-BR3 cells developed more experimental brain metastases, in a significantly shorter time than in mice injected with the original MDA-231-GFP cells. Significantly more endothelial vessels/unit area (microvessel density, MVD) were counted in sections of brain metastases of the 231-BR3 cells, compared with the MVD scored in metastases of MDA-231-GFP cells. Vascular endothelial growth factor (VEGF-A) is a key angiogenic factor in pathological situations that involve neovascularization as well as enhanced vascular permeability, with a high correlation between VEGF expression and peritumoral edema and vascularity of brain tumors. The brain metastasis-selected cells expressed significantly more VEGF-A than the original MDA-231-GFP cells when cultured in normoxic conditions. Mice injected with 231-BR3 cells were treated orally with an inhibitor of VEGF receptor tyrosine kinases (PTK787). The MVD recorded in brain metastasis samples was significantly lower in mice treated with the inhibitor. The metastatic brain tumor burden was also significantly reduced, and survival time increased, although the difference was not statistically significant. We conclude that elevated VEGF-A expression contributes to the ability of breast cancer cells to survive and grow as brain metastases. Targeting endothelial cells with a VEGF-receptor specific tyrosine kinase inhibitor reduced angiogenesis and restricted the growth of the brain metastases.

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# IDENTIFYING PROTEIN INTERACTORS OF BREAST CANCER METASTASIS SUPPRESSOR 1, BRMS1

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The focus of this study is to better understand the biology behind the metastasis suppression via BRMS1, a newly identified metastasis suppressor gene. BRMS1 is a protein with a glutamic acid rich N-terminus, coiled-coil domain, an imperfect leucine zipper and nuclear localization signals. It is expressed almost ubiquitously in human tissues at varying levels. Also it is very highly conserved across species (The murine ortholog is 95% identical at amino acid level). Sub-cellular fractionation and fluorescence immuno-cytochemistry has indicated that it localizes to nucleus. BRMS1 is shown to restore homotypic gap-junctional communication, possibly by restoring the connexin 43 expression. These observations make BRMS1 a very important gene to study. Our hypothesis is that it may be involved in transcription regulatory complex.

To reveal the BRMS1 suppression mechanism, it it was necessary to identify if there are any proteins that interact with it. Yeast two hybrid screen was performed using BRMS1 as a bait and human mammary gland library as a prey. We found 8 confirmed positive genetic interactors of BRMS1. These are MRJ (Hsp 40 related chaperone), CCG1 (a protein essential for progression of G phase), SMTN (cytoskeletal protein specific to smooth muscles), FLJ00052 (EST, putative ORF), KPNA5 (karyopherin alpha5), Nmi (interacts with N-myc, STAT, C-myc), BAF 57 (BRG1 associated factor) and RBP1 (Rb binding protein).

BRMS1-RBP1 interaction was further confirmed at cellular level by immunoprecipitation, using cell lines constitutively expressing epitope tagged BRMS1. Furthermore a rough deletion mapping was carried out to get a clue of the domain of BRMS1 interacting with RBP1. The results suggest that the interacting domain possibly lie between amino acids 94-164. Currently we are exploring the relevance of this interaction with respect to metastasis and cell cycle. Also the other interactors are being evaluated by co-immunoprecipitation.

Our previous findings that BRMS1 is involved in suppression of human breast cancer and melanoma metastasis make the identification of BRMS1-related regulatory and signaling pathways an important task. Such findings will be helpful in prognosis as well as identification of new therapeutic targets for cancer metastasis.

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# ANALYSIS OF METASTASIS USING IN VIVO IMAGING

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We propose that chemotactic responses to blood vessels enable metastatic tumor cells to enter the vasculature during metastasis. Studies comparing the metastatic rat mammary adenocarcinoma cell line MTLn3, with a nonmetastatic cell line, MTC, derived from the same original rat 13762NF mammary tumor, indicate that MTLn3 cells are chemotactic to epidermal growth factor (EGF). This raises the possibility that metastatic cells might show stronger polarization and chemotaxis in vivo to endogenous gradients of chemoattractants. Generation of cells labeled with green fluorescent protein (GFP) enabled us to examine the behavior of these cells in vivo, imaging cells in living tumors in animals. Analysis of cells leaving the primary tumour indicates that highly metastatic cells are able to polarise more effectively towards blood vessels while poorly metastatic cells fragment more often when interacting with blood. In addition, there appear to be greater numbers of host immune system cells interacting with metastatic tumours.

To directly measure invasion in vivo, we developed an assay for measuring the number of tumor cells that directly invade into needles inserted into the primary tumor. Needles containing chemoattractants can be used to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor in a live rat as a pure population suitable for further analysis. The most efficient cell collection requires the presence of chemotactic cytokines, such as epidermal growth factor and serum components, and occurs with 15-fold higher efficiency in metastatic tumors compared with nonmetastatic tumors.

Orientation of tumor cells during chemotactic responses to ligands such as EGF begins with lamellipod extension. Evaluation of some of the downstream events in lamellipod extension indicates: (1) plasma membrane distribution of the EGF receptor is uniform but internalized receptor accumulates on the side of the cell closest to the source of EGF; (2) the alpha p110 isoform of PI-3 kinase is required; and (3) protrusion of the lamellipod relies upon the combined actions of the Arp2/3 complex and cofilin for generation of filamentous actin. Increased expression of the EGF receptor on the surfaces of tumor cells may enhance the sensitivity of tumor cells to gradients in vivo.

We are now extending these studies to transgenic mice as well as testing the roles of EGF receptor family members in metastasis. Expression of GFP in specific cell types in the mammary gland provides a view of how different cell types interact in the primary tumor. Increasing expression of the EGF receptor enhances intravasation. These studies are important for determining mechanisms by which tumor cells enter the blood during metastasis.

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# A SHIFT IN EXPRESSION OF COMPONENTS OF LIPID RAFTS IN ER-NEGATIVE BREAST CANCER

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Several recent approaches using DNA arrays to screen for specific breast cancer subtypes revealed significant differences in gene expression profiles between ER positive and negative breast carcinoma. Since we have found dramatic overexpression of eN (ecto-5'-nucleotidase or CD73), a component of lipid rafts, in ER- breast cancer cell lines, we undertook investigation of the functional significance of eN in more aggressive and metastatic breast cancer cells. Toward this end, we have analyzed selective groups of membrane proteins as well as transcription factors that potentially may co-express with, or be involved in, the regulation of expression of eN. As a cellular model mimicking transition to ER-negative breast cancer we used the MCF-7 cell line and its two unrelated derivatives: drug resistant Adr2 and c-Jun transformed 2-33 clone. Our directed expression profiling revealed a remarkable coordinate shift in expression profiles of membrane proteins, such as MDR-1, caveolin-1, CD44, integrin beta 1 and signaling proteins, such as FAK, Lyn, Src, Lck, trimeric Gi-2. Among the cell surface antigens previously established as markers of breast crcinoma we have observed significant downeregulation of CD24 and uPAR. Since eN, CD24, uPAR and most of signaling molecules surveyed in this study are components of lipid rafts, the results from our limited profiling also suggest that there is a major remodeling of lipid rafts during transition to ER negative breast cancer. To investigate whether the change in lipid raft composition would have functional significance, we have clustered lipids rafts with lectin Concanavalin A and observed dose- and time-dependent increase in phosphorylation of c-Src and FAK in ER negative cells but not in MCF-7 cells. Furthermore, upon clustering of membrane glycoproteins we have also observed a significantly increased association of lipid rafts with cytoskeleton which was completely reversed by Latrunculin A, an inhibitor of actin polymerization. Thus, our results suggest that during transition to more aggressive breast cancer phenotype there is a significant structural re-organization of lipid rafts that leads to altered signaling properties of this membrane domain.

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# MORPHOLOGICALLY SIMILAR STROMAL CELLS ASSOCIATED WITH BENIGN AND MALIGNANT MAMMARY EPITHELIAL TUMORS DISPLAY DIFFERENT IMMUNOHISTOCHEMICAL AND MOLECULAR PROFILES

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Our previous studies on paraffin embedded tissues from patients with mammary and cervical carcinomas revealed high frequencies of independent and concurrent loss of heterozygosity (LOH) in microdissected epithelial (EP) tumor cells and adjacent or distant stromal (ST) cells. To confirm previous findings on a larger scale and wider spectrum, the current study attempted to compare the immunostaining pattern and the genetic profile in EP and ST cells microdissected from infiltrating syringomatous adenomas and tubular carcinomas, which are two different pathological entities, but with similar reactive background stroma.

Serial sections were made from formalin-fixed, paraffin-embedded mammary tissues from patients with above lesions, and immunostained with a panel of different antibodies. The immunostaining patterns in both the EP and ST components between two lesions were compared. Morphologically similar EP and ST cells in these lesions were microdissected for DNA extraction and assessments for LOH and microsatellite instability (MI), using PCR amplification with a panel of DNA markers at 6 different chromosomes. The frequency and pattern of LOH and MI in samples of two lesions were compared.

The cells from these two lesions displayed a substantially different immunostaining pattern to a majority of the antibodies tested, including those to tumor suppressor gene products, blood vessel components, extracellular matrix molecules, and proliferation-associated proteins. Also, both the EP and ST cells from these two lesions displayed a substantially different frequency and pattern of LOH and MI at multiple chromosomal loci, including 3p, 11p, 13p, 13q and 16q. There was no distinct LOH or MI with multiple DNA markers at chromosome 17p in the ST cells of either lesion, however.

These findings suggest that morphologically comparable ST cells associated with the benign and malignant EP lesions are bio-functionally and genetically different, but closely related with those in their EP counterparts. These findings also suggest that the functions of ST cells in both lesions are not directly subject to regulation by the p53 gene.

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# INVOLVEMENT OF HUMAN CYR61 IN HEREGULIN INDUCTION OF BREAST CANCER TUMOR PROGRESSION

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The growth factor Heregulin (HRG), expressed in about 30% of breast cancer tumors, induces tumorigenicity and metastasis of the breast cancer epithelial cells MCF-7. In search for HRG downstream effector molecules, we have identified that Cyr61, an angiogenic factor and a member of the CCN family of genes, is differentially expressed in ER-negative, HRG-overexpressing cells. We have also found that Cyr61 is overexpressed in invasive and metastatic breast cancer cells and tissues. To determine whether Cyr61 indeed plays a role in HRG induction of breast tumor progression, we introduced an antisense Cyr61 cDNA into HRG-transfected MCF-7 cells (MCF-7/HRG) to block Cyr61 expression. Our results show that blockage of Cyr61 expression suppresses the aggressive phenotype of the MCF-7/HRG cells by inhibiting cell proliferation, preventing anchorage-independent growth, and suppressing the invasive potential of the cells in vitro. Moreover, these cells regain estrogen (E2)-dependence and anti-E2 sensitivity. Most importantly, we observed a marked reduction of tumor formation and tumor size in vivo. We further investigated whether Cyr61 is sufficient to bypass HRG to induce breast tumorigenicity and cancer progression. We demonstrate that Cyr61 is sufficient to induce E2-independence and anti-E2 resistance in MCF-7 cells. Cyr61-transfected MCF-7 (MCF-7/Cyr61) cells are anchorage-independent and capable of forming Matrigel outgrowth patterns in the absence of E2. Furthermore, MCF-7/Cyr61 cells are tumorigenic in athymic nude mice. We demonstrate that one of the mechanisms by which Cyr61 induces breast tumorigenicity and cancer progression is possibly mediated via a specific activation of the MAPK and PI3K signaling, and the activity of MMP-9. Our results demonstrate that Cyr61 is a tumor-promoting factor and a key regulator of breast cancer progression, and that Cyr61 should be deemed a potential target in developing therapies to breast cancer.

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# POSTSURGICAL MURINE MODEL OF BREAST CANCER DORMANCY

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Tumor metastasis, the spread of cancer from a primary tumor to distant sites in the body, is the major cause of treatment failure and death for breast cancer patients. Even in patients who have had successful surgical treatment of the primary tumor, disseminated tumor cells may lie dormant posing a continual threat of metastatic disease. Animal models are needed to understand the status of dormant cells and to test therapeutic strategies targeted to this phase of the metastatic process.

The purpose of this research is thus to develop and characterize a murine model in which dormancy and the behavior of breast cancer following surgery can be studied. Our goal is to establish primary tumors in mouse mammary fat pads, surgically remove them to mimic clinical treatment of primary breast cancer, and then assess the status of any disseminated tumor cells after surgery.

Human MDA-MB-435 and MDA-MB-468 mammary carcinoma cell lines were transfected with GFP cDNA to produce a stable cytoplasmic marker, allowing gross metastases to be detected by epifluorescent microscopy. It was established that transfection and/or GFP expression did not alter the *in vitro* or *in vivo* growth kinetics of these cell lines. It was necessary to develop a method to detect micrometastases and single tumor cells. A number of immunohistochemical markers were assessed. The monoclonal antibody Mitochondria Ab-2 was found to be capable of reliably detecting human tumor cells in murine tissues. This antibody stains the mitochondria of normal and malignant human cells and was equally sensitive in detecting both the MDA-MB-435 and MDA-MB-468 cell lines (GFP-expressing and non-transfected), without cross-reactivity to murine tissues.

The development of this model will now permit us to locate and identify micrometastases and single cell metastases and to determine their dormant status by proliferation (Ki-67 antigen) and apoptotic (TUNEL assay) indices. The ability to profile post-surgical metastatic growth will permit us to investigate mechanisms responsible for dormancy in breast cancer.

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# DEMONSTRATION OF TUMOR HYPOXIA IN HUMAN BREAST CANCER

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Laboratory studies of tumor microenvironments suggest a significant role of hypoxia in tumor invasion, angiogenesis and metastases. Clinical studies in cervical carcinoma and head and neck cancer show that tumor hypoxia is associated with poor prognosis. However little is known about hypoxia in breast cancer due to lack of suitable methods of hypoxia detection. The aim of this study is to demonstrate the presence of hypoxia in human breast cancer using a clinically suitable method.

Patients and Methods: Pimonidazole is a 2-nitroimidazole that covalently binds to metabolically active cells that are at $pO2 \leq 10$ mm Hg. Pimonidazole adducts can be detected by monoclonal and polyclonal antibodies. Investigational New Drug (IND No. 36, 783) clearance for pimonidazole as a marker for human tumor hypoxia has been obtained. Patients with biopsy confirmed breast carcinoma are enrolled in two Institutional Review Board approved studies of tumor hypoxia after informed consent is obtained. Thirty-seven patients were infused with pimonidazole in saline at a dose of 0.5 gm/m2. Breast tumor biopsies were obtained the next day at the time of lumpectomy or mastectomy. Biopsies were formalin-fixed, paraffin-embedded, sectioned, and immunostained for pimonidazole adducts (hypoxia). A semiquantitative scoring system (grade 0-4) was used to quantify tumor hypoxia. TNM staging system (T1-T4) was used to classify primary tumors.

Results: Presence of tumor hypoxia was demonstrated in 27/37 patients (73%) and was absent in 10 patients (27%). A wide range of hypoxia was observed from Grade 0 to Grade 4 in T1-T4 tumors. No adverse effects have been observed from the study procedures.

Conclusions: This is the first demonstration of tumor hypoxia in human breast cancer using pimonidazole. Pimonidazole infusion and tumor biopsy at the time of patient's planned surgery provide a practical, clinically applicable hypoxia assessment method. This research is important as it introduces a practical method to study hypoxia as a prognostic factor in breast cancer, to study relationships of hypoxia to other biomarkers of invasion and metastases, and can be useful in selecting patients for future hypoxia based treatments.

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# INVOLVEMENT OF HEPARANASE IN BREAST CANCER PROGRESSION

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Heparanase, degrading heparan sulfate (HS) at specific intrachain sites, affects the integrity and functional state of tissues and thereby fundamental normal and pathological phenomena involving cell migration and response to changes in the extracellular matrix. Heparanase also releases angiogenic factors and accessory fragments of HS from the tumor microenvironment and hence induces an angiogenic response *in vivo*. Our research was undertaken to investigate the involvement of heparanase in breast cancer progression, taking into account its ability to promote tumor cell invasion, vascularization and survival. The pattern of heparanase expression in breast tumor specimens was determined by *in situ* hybridization and immunostaining. Breast carcinoma cells were transfected with the heparanase cDNA and evaluated in mice for an effect on tumor growth, vascularization and dissemination. Transgenic mice over-expressing the heparanase gene were generated and tested for alterations in mammary gland morphogenesis and remodeling. Human mammary carcinoma express the heparanase mRNA and protein in both the *in situ* and invasive components of ductal and lobular origins. Normal breast tissue expresses little or no heparanase. Primary tumors produced by MCF-7 breast cancer cells over-expressing the human, mostly intracellular enzyme, exhibited a 3-4 fold accelerated growth and size compared with tumors produced by control mock-transfected cells. A more pronounced effect on tumor size and vascularity was observed with MCF-7 cells transfected with a secreted and membrane bound form of heparanase, recently cloned in our laboratory, indicating that cellular localization of heparanase plays an important role in breast cancer progression. Translocation and secretion of heparanase in MCF-7 cells were enhanced in response to estrogen that also stimulated heparanase promoter activity. Mammary glands of transgenic mice over-expressing the heparanase enzyme exhibited precocious branching and widening of ducts, as well as basement membrane disruption and early signs of hyperplasia. Serum free medium conditioned by MDA-435 breast carcinoma cells was identified as a suitable source for characterization and purification of the putative protease, converting the 65 kDa latent heparanase into an active 50 kDa enzyme. Processing and activation of the latent enzyme occurred also on the cell surface, indicating that heparanase cleavage is a cell surface event. The unexpected identification of a single predominant functional heparanase suggests that the enzyme is a promising target for drug development. A ribozyme targeting approach was applied to suppress heparanase expression in tumor cells. A marked reduction in heparanase activity and cell invasion was observed with ribozyme transfected MDA-435 cells. Lung colonization of breast carcinoma cells was inhibited by fragments of laminaran sulfate, which inhibit heparanase activity. We conclude that heparanase plays a role in critical steps of breast cancer progression. It is hoped that identification of the sugar residues in HS adjacent to the heparanase cleavage site, as well as crystallization and analysis of the 3D-structure of the enzyme will lead to a rational design of highly specific heparanase inhibitors that will be tested in animal models and breast cancer patients.

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# FGF-2 INDUCES BREAST CANCER SURVIVAL ON FIBRONECTIN: A PARADIGM FOR DORMANCY

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Micrometastatic cells in the bone marrow of newly diagnosed breast cancer patients are resistant to adjuvant chemotherapy. These cells can remain dormant for years before they begin to proliferate and result in recurrent disease. Elements of the bone marrow microenvironment are responsible for conferring survival signals to these cells. We have previously demonstrated that basic fibroblast growth factor (FGF-2), a protein abundantly expressed and deposited in the bone marrow stroma, induces cell cycle arrest in breast cancer cells and thus, may be a likely candidate for playing a role in dormancy.

We investigated the effects of FGF-2 on the clonogenic growth of breast cancer cells on plates coated with fibronectin, one of the primary matrix proteins present in the bone marrow stroma. FGF-2 10 ng/ml completely inhibited the formation of 24-30 cell stage colonies after five days of culture in MCF-7 and T-47D, two well differentiated breast cancer cell lines, but had no effect on MDA-MB-231, a highly dedifferentiated cell line representative of cells unlikely to remain dormant in bone marrow. The FGF-2-treated MCF-7 and T-47D cells remained in the 4-8 cell stage on fibronectin for up to 8 days, the duration of our follow-up. Survival in the presence of FGF-2 was only marginal, however, when incubated on collagen I, collagen IV or laminin I. The survival of MCF-7 and T-47D cells incubated with FGF-2 correlated with an increase in the expression of integrin alpha-5. FGF-2 had no effect on integrin alpha-5 levels in MDA-MB-231 cells. Anti-alpha-5 blocking antibody resulted in a 60% inhibition of 24-30 cell stage colony formation in MCF-7 cells while no effect was observed with anti-alpha-3 antibody. Similarly, blocking antibody to integrin alpha-5 diminished the survival of 4-8 cell clusters by 60 % after 5 days in the presence of FGF-2. These experiments demonstrate that FGF-2 can arrest well differentiated breast cancer cells on various substrata and upregulate integrin alpha-5. In these arrested cells, the upregulation of the fibronectin receptor may provide a survival advantage in the bone marrow microenvironment. This suggests a possible paradigm for dormancy of micrometastatic cells in bone marrow and points to a potential therapeutic target in integrin alpha-5.

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